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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*

Selenium is an essential nutrient that may have an important preventive action in prostate cancer. We have identified a human gene in prostate cells, designated hSP56, the mouse homologue of which may mediate selenium's growth inhibitory effect in vitro and anti-cancer effect in vivo. hSP56 is expressed by LNCaP but not by PC-3 and is reversibly downregulated by androgen. We hypothesize that hSP56 protein may play a role in the anti-cancer effects of selenium in the prostate gland. To test this hypothesis we will 1) study the molecular properties of hSP56 protein, including its binding of selenium, 2) study the role of hSP56 in the prostate cancer phenotype in vitro using gain-of-function and loss-of-function approaches, 3) investigate the effect of overexpression or downregulation of hSP56 on in vivo tumor growth in SCID mice, and 4) determine the distribution of hSP56 expression in primary human tumors and in adjacent normal prostate cancer cells. This work will lead to a new understanding of prostate cancer regulation and the role of selenium in normal and malignant prostate growth and spread.

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INTRODUCTION

The essential trace element selenium was first associated with cancer risk more than two decades ago. Whereas a deficiency in dietary selenium is associated with an increased incidence of several cancers, including prostate, dietary selenium supplementation has been shown to reduce the incidence of prostate and other cancers. We recently discovered that a human selenium binding protein, hSP56, is expressed by the slowly growing androgen sensitive LNCaP cell line but not by the more rapidly growing, highly metastatic androgen insensitive PC-3 cell line. Treatment of LNCaP cells with androgen results in downregulation of hSP56 expression and a concomitant increase in cell growth, suggesting that hSP56 may mediate the anti-cancer effect of selenium in humans. We hypothesize that hSP56 is a growth regulating protein that modulates the phenotype of human prostate cancer and may mediate the anti-cancer effect of dietary selenium. The study will demonstrate the location of selenium binding on the hSP56 molecule. It will also demonstrate that changes in hSP56 expression and selenium alter the *in vitro* and *in vivo* phenotype of human prostate cancer cells. LNCaP, cells that express endogenous hSP56 constitutively, will be transfected with an inducible antisense construct, thereby allowing hSP56 to be downregulated. These cells will be used in loss-of-function experiments, that is, examining the *in vitro* and *in vivo* phenotype of cells in which hSP56 has been downregulated. PC-3 cells, which do not express hSP56, will be transfected with an inducible hSP56 sense construct, thereby allowing gain-of-function experiments, that is, studying the effect of increased hSP56 expression on the PC-3 phenotype. Lastly, using *in situ* hybridization and immunohistochemistry, the pattern of hSP56 expression in primary tumors and bone marrow metastases will be elucidated.

BODY

Experimental Procedures- New Techniques Developed in the First Year

Construction of bacterial expression plasmids-- A cDNA clone that contains the largest hSP56 cDNA of 1721 bp was isolated. It contains a complete open reading frame of hSP56 with more sequence in both 5' and 3' untranslated regions than our original clone (NCBI accession number BC009084). The original hSP56 cDNA cloned by Chang et al (NCBI accession number U29091) is 1429 bp in length with minimal 5' and 3' untranslated sequences. The bacterial expression vectors used were pTrcHisA and pTrcHisB for producing recombinant hSP56 without or with N-terminal $(His)_6$ fusion (InVitrogen). The complete coding cDNA of hSP56 protein was cloned into the *Nco* I and *Eco* RI sites of pTrcHisA for non-fusion expression and into *Bam*H I and *Eco* RI sites of pTrcHisB fused in-frame with the N-terminal $(His)_6$ sequence for producing $(His)_6$-fusion protein in *E. coli*. For N-terminal $(His)_6$-fusion protein expression, the translational initiation codon was removed. The DNA fragments for both $(His)_6$ fusion and non-fusion expression were generated by high fidelity PCR reaction using the cDNA clone of hSP56-BC009084 as template. Restriction fragments containing hSP56 coding sequences were obtained by digestion with respective enzymes and gel purified before the ligation reaction. The PCR primer pairs were SEBP1 (5'- CGCGGATCCGGCTACGAAATGTGGGAATTG-3') and SEBP2 (5'- GCGGAATTCATACAATCCAGATGTCAGAG-3') for $(His)_6$ fusion construct (pTrcHisB-hSP56nHis) and SEBPN3 (5'-CATGCCATGGCTACGAAATGTGG -3') and SEBPC4 (5'-

CCGGAATTCAACAAGCAACAGTGGTCAG-3') for non-fusion expression (pTrcHisA-hSP56). These bacterial expression constructs are all verified by DNA sequencing to ensure the fidelity of the subcloning. The resulting expression constructs pTrcHisA-hSP56 and pTrcHisB-hSP56nHis were transformed into *E coli* TOP10F' by electroporation. Host cells harboring the expression plasmids were grown in LB broth with 100 μg/ml ampicillin. To express the recombinant N-terminal $(His)_6$ fusion human selenium-binding protein (rhSP56nHis) of 503 amino acid residues or the recombinant human selenium-binding protein (rhSP56) of 472 residues, 1 mM of IPTG was added to the culture medium to induce expression.

Construction of mammalian cell expression plasmids-- The mammalian expression vector was pcDNA4/TO/mycHisA (InVitrogen). The hSP56 cDNA was excised out from vector pSPORT6 which contains hSP56-BC009084 cDNA fragment without the putative 3' SECIS sequence and subsequently was ligated into *Hind* III/*Eco* RI sites of pcDNA4/TO/mycHisA in the multiple cloning sequences.

Construction of SECIS functional assay plasmid-- A mammalian expression vector containing human type I deiodinase (107-112-D10) was cleaved with *Hind* III and *Not* I to remove the functional SECIS sequence and replaced with a 265 bp DNA fragment from the 3' UTR of hSP56 (107-112-D10/hSP563UTR). Oligonucleotide primer SECISP1 (5'-CCCAAGCTTTAGACTCCACCCTCATCACCC-3') and SECISP2 (5'-ATAGTTTAGCGGCCGCGAAGGACAGGGTTACGAGTT-3') corresponding to positions 1467-1484 and 1685-1704 of hSP56 BC009084 were synthesized and used in PCR amplification. The PCR primers were designed to include *Hind* III and *Not* I sites at their termini. High fidelity PCR reaction with primers SECISP1 and SECISP2 using hSP56-BC009084 plasmid DNA as template generated DNA fragment of the hSP563UTR, and this DNA was tested for its potential selenocysteine insertion function after cleaved with restriction enzymes inserted into the *Hind* III and *Not* I sites of expression construct 107-112-D10 .

Transient Expression of hSP56 in prostate cancer cells-- Prostate carcinoma cell lines LNCaP and PC-3 were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum. Twenty-four hours prior to the transfection treatment, the cells were plated in 2 ml of medium in 6-well plates at a density of 2×10^5 cells/well. For transient expression of hSP56, 5 μg of expression construct DNA was added to the cells with Lipofectin reagent from LifeTechnologies following the manufacturer's protocols.

Deiodinase assay (based on Kumaraswamy)-- Transient transfection of SECIS functional assay plasmids was done by the calcium phosphate method. Human embryonic kidney (HEK293) cells were used as host cells. Cells were plated onto 60 mm culture dishes in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum three day before the transfection. Cells were transfected with 10 μg of 107-112-D10/hSP563UTR and 4 μg of pUHD15 plasmid that encodes a protein necessary for transcriptional activation of the 107-112-D10 promoter. An additional 3 μg of an expression vector containing the human growth hormone cDNA under the control of the hamster sarcoma virus thymidine kinase promotor were co-transfected to allow quantification of the transfection efficiency. As a positive control, the expression construct 107-112-D10 containing the functional SECIS of human type I deiodinase was used. Cells were harvested two days after transfection by scraping and were lysed by sonication. Cells were

washed and resuspended in 0.1 M potassium phosphate, pH 6.9, containing 1 mM EDTA, 0.25 M sucrose, and 10 mM dithiothreitol. The active deiodinase was assayed by its ability to 5'-deiodinate ^{125}I reverse T3. Total protein of 10 μg was added to the reaction containing 1 μM ^{125}I reverse T3, 10 mM dithiothreitol in a volume of 300 μl. After incubation at 37 °C for 30 min., ^{125}I release was quantified. The detected deiodinase activity was normalized to the amount of growth hormone secreted into the medium. Each expression construct was tested in duplicate transfection, and each sample was assayed twice.

Progress Made On Each Task

TASK 1: Study the cellular and molecular properties of hSP56 protein

The first cDNA sequence of hSP56 identified by Chang *et al.* and confirmed by us contains only minimum sequences in both 5' and 3' untranslated regions. The deposition of DNA sequencing results of the Human Genome Project into the NCBI databases provided hSP56 cDNA clones with better annotation. The addition of more than 250 bp of 3' untranslated sequence suggested the possibility that hSP56 mRNA contains a potential **selenocysteine insertion sequence** (SECIS) in its 3' UTR. In other mammalian selenoproteins, selenium is incorporated into the protein by a specific mechanism. It involves a UGA codon and a 3' regulatory SECIS sequence in the mRNA, tRNA charged with selenocysteine, and other protein factors. If this were the case with hSP56, that is, if hSP56 were a selenocysteine-containing protein, then much or our proposed research under Task 1 would have to be redesigned. Therefore, we took advantage of the well established SECIS activity assay and prepared a mammalian expression construct plasmid that contains the open reading frame of the human deiodinase sequence and replaced its own functional SECIS sequence with the DNA sequence of hSP56 3' UTR. This method has been used to evaluate whether potential SECIS sequences function to direct the insertion of a selenocysteine residue, which is required for the enzyme to be active (Figure 1). Importantly, we found no deiodinase activity in cells transfected with our test construct DNA, thus proving that hSP56 is not a selenocysteine-containing protein, but rather a selenium binding protein, validating our Task 1 experiments.

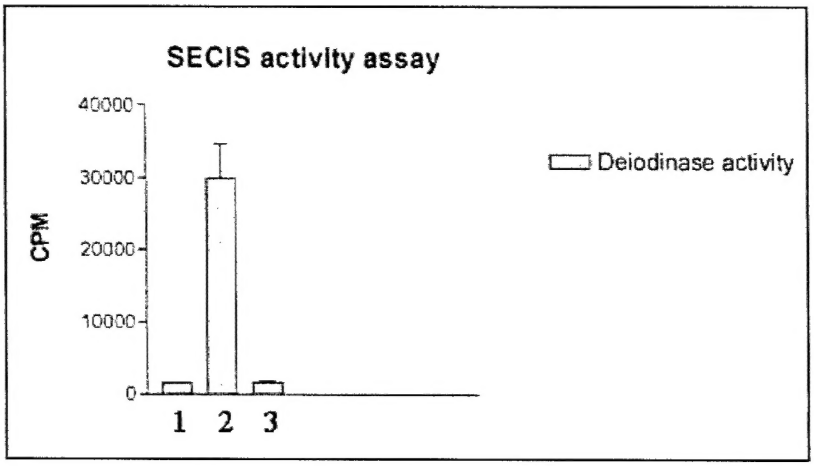

Figure 1. Deiodinase assay shows that 3' UTR of hSP56 does not contain an SECIS. 1) Negative control; 2) Positive control; 3) hSP56 3'UTR shows no activity.

TASK 2: Investigate the role of hSP56 and selenium in the prostate cancer phenotype *in vitro*

We began gain-of-function studies involving the transient transfection and over-expression of hSP56 in prostate cancer cells. We discovered that expression of hSP56 triggers apoptosis in PC-3 cells, In order to begin to study *hSP56* through "gain-of-function" experiments, we transfected PC-3 cells, in which *hSP56* expression is low to absent, and subjected them to biochemical selection. However, the cells stably transfected with *hSP56* failed to proliferate, unlike that vector-transfected controls, suggesting a growth inhibiting action of hSP56 protein on PC-3 carcinoma. Transient transfection of PC-3 with *hSP56* revealed that expression of hSP56 protein resulted in the induction of apoptosis (Figure 2). Cells were grown to 65% confluence and then treated with paclitaxel (to induce apoptosis-positive control) or transfected with the expression vector pcDNA4-*hSP56* or control vector. After 48 hr, cells were lysed and **poly(ADP-ribose) polymerase (PARP)** protein was detected by SDS-PAGE and western blot. The 116 kDa intact PARP protein is hydrolyzed by caspase-3 to an 85 kDa fragment upon activation of the apoptotic pathway and, therefore, serves as a marker for apoptosis. As seen in Figure 2, lane 1, no apoptosis was detected in control cells (intact 116 kDa PARP protein). However, a prominent 85 kDa band, indicative of apoptosis, was detected in lysates of paclitaxel treated cells as expected (lane 2) and, importantly, in cells transfected with hSP56 (lane 3). Western blot using our anti-hSP56 antibody confirmed expression of the hSP56 protein in the transfected cells (not shown). These results provide strong evidence that induction of apoptosis may be one part of the anti-cancer action of hSP56.

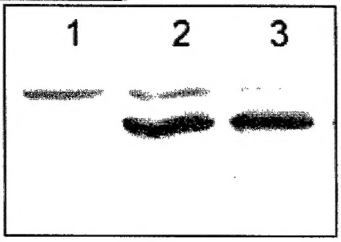

Figure 2. Expression of hSP56 triggers apoptosis in PC-3 cells. Western blot probed with anti-PARP Ab. Lane 1, control cells; lane 2, cells treated with 50 ng paclitaxel/ml; lane 3, cells transfected with *hSP56*. Note prominent 85kDa band indicating activation of apoptotic pathway.

TASK 3: Determine effects of altered hSP56 expression and dietary selenium on tumor growth and spread *in vivo*.

- We began training Dr. Wang and Mrs. Wellenstein in animal surgery procedures and tumor implantation.
- We initiated baseline growth kinetics studies.

TASK 4: To carry out a pilot study to determine the distribution of hSP56 in primary tumors, metastatic foci and normal prostate tissue and correlate with Gleason grade

We determined that our antibodies to hSP56 protein are very useful for immunohistochemical detection of the protein, an important aim of the proposed research. We transplanted LNCaP cells orthotopically into the ventral prostate of SCID mice. After tumors had formed, they were excised, fixed, embedded in paraffin and mounted onto glass slides. After deparaffinization the slides were incubated with specified dilutions of anti-hSP56 antibody. Then biotinylated anti-

rabbit secondary antibody was applied followed by avidin-biotin peroxidase complex. After washing in PBS, the color was developed with diaminobenzidine. Hematoxylin was used as a counterstain. Figure 3 shows intensely staining hSP56 protein in the cytoplasm of the LNCaP cells. The cells differ in the apparent amount of hSP56 protein expressed. The murine tissue compressed by the tumor does not stain for hSP56, illustrating the specificity of the antibody. This specificity is seen especially clearly in Figure 4. In this photomicrograph, hSP56-expressing LNCaP cells, on the left, are seen invading mouse prostate glandular tissue, which is decidedly negative. The ability of this highly specific antibody to differentiate among cells expressing hSP56 at different levels will greatly enhance our immunohistochemistry studies of human tumors and metastatic foci.

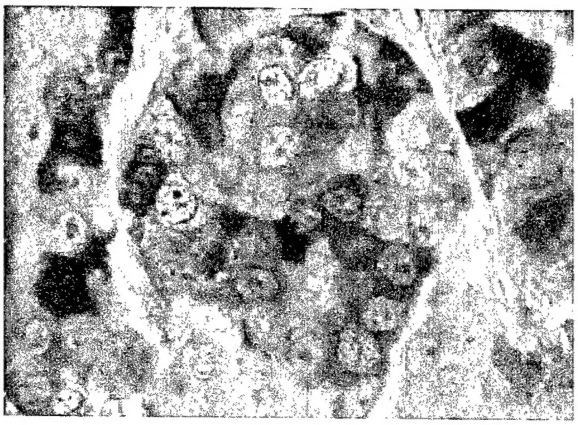

Figure 3. Immunohistochemical detection of hSP56 protein in LNCaP tumor cells.

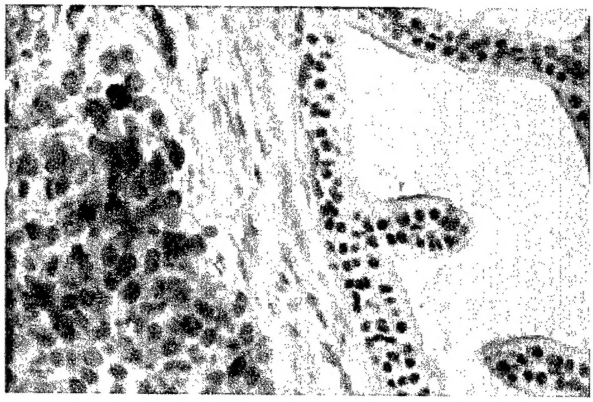

Figure 4. hSP56 protein in LNCaP tumor cells (left) invading SCID mouse prostate (right).

KEY RESEARCH ACCOMPLISHMENTS

- Construction of bacterial expression plasmids
- Construction of mammalian cell expression plasmids
- Construction of SECIS functional assay plasmid
- Transient Expression of hSP56 in prostate cancer cells
- Deiodinase assay
- Discovered more 5' and 5' UTR sequence of hSP56 cDNA
- Proved that hSP56 is not a selenocysteine-containing protein
- Discovered that transient over-expression of hSP56 induces apoptosis in PC-3 cells
- Demonstrated that our antibodies to hSP56 gave excellent results in immunohistochemistry

REPORTABLE OUTCOMES

- Manuscript describing above results is nearing completion
- Applied for funding from NIH

CONCLUSIONS

The research is still in it early stages. The main conclusion reached thus far is that hSP56 is not a selenocysteine-containing protein. Therefore, it must bind selenium in a novel way not yet known in biochemistry. Our Task I experiments should allow us to elucidate this. Other conclusions cannot be made at this stage of the project. There have been no major problems.

REFERENCES

Kumaraswamy E., *et al.* J Biol Chem. 275(45):35540-7, 2000.